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J Immunol. 2016 March 15; 196(6): 2457–2465. doi:10.4049/jimmunol.1501476.**LAMP-2C inhibits MHC class II presentation of cytoplasmic antigens by disrupting chaperone-mediated autophagy**Liliana Pérez^{*}, Shawna McLetchie^{*}, Gail J. Gardiner^{*}, Sarah N. Deffit[†], Delu Zhou[‡], and Janice S. Blum^{*}^{*}Department of Microbiology & Immunology, Indiana University School of Medicine, Indianapolis, IN 46202[†]Medical Sciences Program, Indiana University, Bloomington, IN 47405[‡]Department of Pathology, University of Utah, Salt Lake City, UT 84112**Abstract**

Cells utilize multiple autophagy pathways to sequester macromolecules, senescent organelles, and pathogens. Several conserved isoforms of the lysosome-associated membrane protein (LAMP)-2 regulate these pathways influencing immune recognition and responses. LAMP-2A is required for chaperone-mediated autophagy (CMA) which promotes Ag capture and MHC class II (MHCII) presentation in B cells and signaling in T cells. LAMP-2B regulates lysosome maturation to impact macroautophagy (MA) and phagocytosis. Yet, far less is known about LAMP-2C function. While *LAMP2A* and *LAMP2B* mRNA were broadly detected in human tissues, *LAMP2C* expression was more limited. Transcripts for the three *LAMP2* isoforms increased with B cell activation, although specific gene induction varied depending on TLR versus BCR engagement. To examine LAMP-2C function in human B cells and specifically its role in Ag presentation, ectopic gene expression was used. Increased LAMP-2C expression in B cells did not alter MHCII expression or invariant chain processing, but did perturb cytoplasmic Ag presentation via CMA. MHCII presentation of epitopes from exogenous and membrane Ags was not affected by LAMP-2C expression in B cells. Similarly, changes in B cell LAMP-2C expression did not impact MA. The gene expression of other *LAMP2* isoforms as well as the proteasome and lysosomal proteases activities were unperturbed by LAMP-2C ectopic expression. LAMP-2C levels modulated the steady-state expression of several cytoplasmic proteins which are targeted for degradation by CMA and diminished peptide translocation via this pathway. Thus, LAMP-2C serves as a natural inhibitor of CMA which can selectively skew MHCII presentation of cytoplasmic Ags.

Introduction

MHC class II (MHCII) molecules, displayed on the surface of APCs, present self and foreign antigenic peptides to CD4⁺ T lymphocytes. The presentation of peptides derived

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from exogenous proteins initiates immunological responses, whereas the presentation of self-derived epitopes is critical for the development of self-tolerance. MHCII $\alpha\beta$ heterodimers are synthesized and co-assembled with invariant chain (Ii) in the endoplasmic reticulum (1). Aspartic and cysteine proteases degrade Ii once this complex reaches the late endosomal compartment known as MIIC, generating CLIP which remains bound to the peptide binding groove of MHCII (2, 3). In the presence of antigenic epitopes, HLA-DM catalyzes the removal of CLIP from the MHCII peptide binding groove and facilitates antigenic peptide binding (4, 5). MHCII-peptide complexes are then transported to the cell surface and presented to CD4⁺ T cells. MHCII classically presents epitopes derived from exogenous Ags that are internalized through endocytosis for processing in endosomes and lysosomes (6). Cell surface and endosomal membrane Ags derived from infectious agents or self-proteins are similarly proteolyzed yielding abundant peptide ligands for MHCII (7, 8). Constitutive and stress-modulated degradation pathways such as macroautophagy (MA) and chaperone-mediated autophagy (CMA) sequester cytoplasmic and nuclear proteins into endosomes and lysosomes, giving rise to roughly 10–30% of the antigenic peptides presented by MHCII.

MA is constitutively active at low levels in most immune cells and can be induced by stress or infection, driving the formation of double membrane vesicles known as autophagosomes which sequester cytosolic contents including pathogens and organelles. Fusion of autophagosomes with lysosomal compartments results in vesicular content degradation giving rise to fragmented macromolecules. This autophagy pathway is important for pathogen elimination, regulation of pattern recognition receptors and the inflammasome, Ag presentation, and T and B cell homeostasis (9–11). CMA provides another pathway for surveillance of the cytoplasm which facilitates MHCII presentation of epitopes derived from specific cytoplasmic Ags. During CMA, select cytoplasmic proteins are targeted and recognized by association with HSC70 and HSP90 (12). This chaperone complex interacts with lysosome-associated membrane protein (LAMP)-2A, driving the translocation of targeted proteins and peptides into the lysosome for processing and in some cases, binding to MHCII. CMA is also modulated during periods of stress such as nutrient deprivation and oxidative stress as well as with cell transformation and aging (13–15).

LAMP-2A belongs to a family of highly homologous type I transmembrane proteins, each with a conserved glycosylated domain extending into the acidic lysosomal lumen. In humans, LAMP-2 (CD107b) protein is derived from a single gene with nine exons. Alternative splicing of exon nine generates three isoforms known as LAMP-2A, LAMP-2B, and LAMP-2C, which differ primarily in the sequence of their transmembrane and cytosolic tail (Fig. 1A). In patients with the inherited disorder Danon disease, deficiencies in LAMP-2 result in impaired MHCII presentation of exogenous but not membrane Ags, as well as disruptions in lysosome biogenesis and phagocytosis (16, 17). LAMP-2A is known to function biologically in lysosomes as the translocator for CMA. During CD4⁺ T cell activation, LAMP-2A protein expression increases and promotes the ubiquitin ligase

Itch and the calcineurin inhibitor RCAN1 degradation via CMA to extend cell signaling (18). In B cells, MHCII-restricted presentation of several cytoplasmic autoantigens is dependent on LAMP-2A expression (19). LAMP-2B by contrast, modulates lysosome

fusion and possibly MA, although its role in Ag presentation is less clear as ectopic expression of this isoform in human B lymphocytes did not alter MHCII presentation (17, 19). Recent publications have implicated LAMP-2C in the uptake and degradation of RNA and DNA molecules (20, 21). However, the importance and role of LAMP-2C in immune recognition remains to be elucidated. In the current study, distinct gene expression patterns for *LAMP2* isoforms were revealed with human B cell activation via BCR crosslinking and TLR ligand exposure. Given increased *LAMP2C* expression in response to B cell activation, the role of this isoform in modulating MHCII Ag presentation and autophagy pathways was determined. LAMP-2C functioned as a novel endogenous negative regulator of CMA which disrupted molecular translocation into lysosomes but did not alter MA. Increased cellular expression of LAMP-2C also skewed MHCII Ag presentation by disrupting cytoplasmic epitope presentation to CD4⁺ T cells.

Materials and Methods

Cell Lines

Human B lymphoblastoid cell lines (B-LCL) PriessGAD and FrevSMA cells have been described (19) and were maintained in IMDM with 10% heat-inactivated FCS, 50 U/ml penicillin, and 50 µg/ml streptomycin. T cell hybridomas recognizing epitopes presented by HLA-DR4, 33.1 (specific for GAD₂₇₃₋₂₈₅), 2.18 (specific for Ig κI₁₈₈₋₂₀₃), 1.21 (specific for Ig κII₁₄₅₋₁₅₉), 17.9 (specific for HSA₆₄₋₇₆), 49.23.2 (specific for an epitope of tetanus toxin), 1736-DR4-3B2 (specific for an epitope of MP1 provided by Dr. D. Canaday, Case Western University), and 50.84.17 (specific for HA₃₀₇₋₃₁₉) were cultured in RPMI-1640 with 10% FBS, 0.1% 2-ME, 50 U/ml penicillin, and 50 µg/ml streptomycin.

Plasmids and Cell Transfection

LAMP2C cDNA was inserted into the vector pcDNA3.1/Zeo (-) via EcoRV and BamHI restriction sites or into the vector pCMV Tag-1 via SacI and SalI restriction sites. PriessGAD B-LCL were transfected with linearized plasmid (10–20 µg/ml) by electroporation (250 V, 950 µF) and incubated on ice 10 min. Transfection of FrevSMA B-LCL was achieved using Xfect Transfection Reagent (Clontech). PriessGAD zeo and PriessGAD 2c were transduced to express the cytoplasmic influenza A matrix protein 1 (MP1) fused to the autophagy protein LC3 using a lentiviral vector provided by Dr. C. Münz (University of Zurich) (22). Drug-resistant cell lines were selected and screened for target gene expression.

RT-PCR

For detecting *LAMP2* isoform expression, a cDNA panel of human tissue was purchased from Clontech. The 5' primer for all three *LAMP2* isoforms was 5'-GAAGGAAGTGAACATCAGCATG-3', the 3' primer for *LAMP2A* was 5'-CTCGAGCTAAAATTGCTCATATCCAGC-3', for *LAMP2B* was 5'-CAAGCCTGAAAGACCAGCACC-3', and for *LAMP2C* was 5'-CTCGAGTTACACAGACTGATAACCAGTAC-3'. The 5' primer for *GAPDH* was 5'-ATGGCACCGTCAAGGCTGAG-3' and the 3' primer was 5'-TGCAGGAGGCATTGCTGATG-3'. Platinum PCR supermix (Invitrogen) was used for

amplification. *LAMP2* cDNAs were amplified for 35 cycles. To detect *SMA* expression, cellular RNA was extracted using RNeasy Mini Kit (Qiagen) and cDNA was generated using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). The 5' primer for *SMA* was 5'-GACTCTGGTCTTCATATCCATACTGCT-3' and the 3' primer was 5'-GCAGTAATAAACGGCTACATCTTCA-3'. *SMA* cDNA was amplified using 2X Reddy Mix PCR Master Mix (Thermo Scientific) for 35 cycles. *GAPDH* cDNA was amplified for 30 cycles. PCR products were resolved on an agarose gel.

Real-Time Quantitative PCR (qPCR)

RNA was extracted from human and mouse tissues or human B cells and used to prepare cDNA. qPCR was performed using the 7500 Fast RT-PCR System (Applied Biosystems) and TaqMan primers for *LAMP2A*, *LAMP2B*, and *LAMP2C* (Supplemental Fig. 1A) or commercially available TaqMan primers for *CD86*, *GAPDH*, or *18S rRNA*. qPCR was performed using TaqMan primers designed for detection of murine *Lamp2a*, *Lamp2b*, and *Lamp2c* (Supplemental Fig. 1A) or commercially available TaqMan primers for murine *Actb* (Applied Biosystems). Gene expression in B cells was normalized to *18S rRNA* or *GAPDH* mRNA levels and presented as an arbitrary fold change (RQ) compared to control samples. Gene expression in tissue samples was presented as mRNA expression relative to *GAPDH* or *Actb* mRNA levels.

Semi-nested qPCR

For detection of *LAMP2C* in human peripheral blood B cells, spleen, or muscle, two rounds of PCR amplification were used. *LAMP2C* cDNA was amplified 10 cycles using 2 μ l of total cDNA, Platinum Taq DNA Polymerase High Fidelity (Invitrogen), and 0.2 μ M of primers for *LAMP2C*. qPCR was performed using TaqMan primers for *LAMP2C* with 0.5 μ l of the first PCR for semi-nested qPCR.

Antibodies

LAMP-1 (H4A3) and LAMP-2 (H4B4) Abs were from the Developmental Studies Hybridoma Bank (University of Iowa). DA6.147 Ab recognizes HLA-DR α chain (P. Cresswell, Yale University). L243 Ab recognizes HLA-DR $\alpha\beta$ dimers and pin1.1 Ab detects Ii. The Ab for GILT was developed in our laboratory. HSC70 and HSP90 Abs were from Enzo Life Sciences, GAD65/67 Ab from Sigma-Aldrich, and cathepsin D Ab from Calbiochem. LC3 and p-I κ B α Abs were obtained from Cell Signaling. Actin and RNase A Abs were from Thermo Scientific. GAPDH Ab was from Millipore. HLA-DQ Ab was from Abcam and HLA-DP and HLA-DO β Abs from Santa Cruz Biotechnology, Inc. were used in flow cytometry. FITC tagged CerClip Ab was used to detect CLIP and FITC labeled HLA-DR Ab to detect HLA-DR $\alpha\beta$ dimers by flow cytometry (BD Biosciences).

Flow Cytometry

For detection of surface CLIP, HLA-DR, -DP, or -DQ, APCs were incubated 1 h on ice with appropriate Ab. APCs were washed with FACS buffer (PBS, 1% BSA, 0.1% NaN₃) and fixed with 1% paraformaldehyde (PFA). For detection of intracellular HLA-DO, APCs were fixed with PFA, permeabilized with 0.1% saponin, blocked with goat serum, and incubated

for 1 h on ice with an Ab to detect HLA-DO followed by FITC labeled rabbit anti-mouse Ab (Sigma-Aldrich). Samples were washed with FACS buffer before analysis. Flow cytometry was performed on BD LSR II and analyzed using FlowJo software (Tree Star).

Immunoblotting

APCs were lysed on ice for 30 min with 10 mM Tris-HCl pH 6.8, 150 mM NaCl, 1% Triton-X 100, protease inhibitor cocktail (Sigma-Aldrich). 50 µg-80 µg protein from lysates were resolved on SDS-PAGE and transferred to nitrocellulose (Bio-Rad). Membranes were probed with specific primary Ab and incubated with goat anti-mouse-, anti-rabbit-, or anti-rat-HRP conjugated secondary Ab (Jackson ImmunoResearch Laboratories, Inc.). Blots were visualized with ECL reagent (Pierce).

Immunoprecipitation

APCs were lysed 30 min on ice with 50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Igepal CA630 (Sigma-Aldrich), protease inhibitor cocktail (Sigma-Aldrich). Lysates were incubated with 5 µg/ml of HSC70 Ab or isotype control overnight at 4°C. Protein complexes were co-immunoprecipitated using µMACS Protein G Microbeads and µ Columns (Miltenyi Biotec). Samples were resolved on SDS-PAGE and analyzed by immunoblotting.

Ag Presentation Assay

To measure endogenous Ag presentation, APCs were co-cultured for 24 h at 37°C with epitope specific T cells. For exogenous Ag presentation, APCs were incubated with synthetic peptides or purified Ag for 4 or 16 h at 37°C and then co-cultured with epitope specific T cells for 24 h at 37°C. For HA Ag presentation, APCs were infected for 16 h with live influenza A X-31, A/Aichi/68 (H3N2) (Charles River) and then co-cultured with epitope specific T cells for 24 h at 37°C. APCs were cultured with T cells at variable APC:T cell ratios. An IL-2 dependent T cell line, HT-2, was used to measure IL-2 produced in response to T cell activation. HT-2 proliferation was evaluated by [³H] thymidine incorporation and quantified by liquid scintillation counting (Wallac Microbeta).

MA Flux

APCs were incubated for 16 h at 37°C +/- 20 µM chloroquine (CQ) (Sigma-Aldrich). Immunoblotting was used to detect cellular LC3II. Protein levels were determined by densitometry using ImageJ software (NIH). LC3II was normalized to GAPDH protein levels. MA flux was determined by subtracting normalized LC3II levels of untreated cells from normalized LC3II levels of CQ treated cells.

Peptide Electroporation

APCs were washed with cold PBS twice and incubated with a GAD₂₇₃₋₂₈₅, KFERQ-GAD₂₇₃₋₂₈₅, or AFERQ-GAD₂₇₃₋₂₈₅ peptide C-terminal labeled with biotin for 5 min on ice. Electroporation (270 V, 125 µF, pulsed twice) was performed using the BIO-RAD gene pulser II to deliver the peptide into the cell cytoplasm. Control cells were incubated with peptides without electroporation. Cells were extensively washed immediately after electroporation and cultured at 37°C for 16 h. APCs were used in an Ag presentation assay

or proteins were resolved by SDS-PAGE to detect GAD₂₇₃₋₂₈₅-biotin/MHCII complexes. For PAGE, samples were transferred to nitrocellulose, incubated with streptavidin-HRP and ECL reagent (Pierce). Protein levels were determined by densitometry using ImageJ software (NIH).

B cell activation

B cells were isolated from healthy adult PBMCs using CD19 MACS beads (Miltenyi Biotec) and incubated at 37°C for 24 h with 20 µg/ml of AffiniPure F(ab')₂ Goat Anti-Human IgG + IgM (H+L) (Jackson ImmunoResearch Laboratories, Inc.), 2 µg/ml of TLR7 agonist R848 (Enzo Life Sciences), or 50 nM of TLR9 agonist CpG ODN 2006 (Invivogen). Cells were harvested, and *LAMP2* and *CD86* mRNA levels measured by qPCR.

ELISA

Cell culture supernatants from human B cells after 24 h of TLR stimulation were analyzed using a standard sandwich ELISA in triplicate to detect IL-6 (Invitrogen).

Protease and Proteasome Assays

For real time analysis of cathepsin B and L activities, the Magic Red™ Cathepsin B and L Kits (Immunochemistry Technologies) were used. Fluorescence was detected using flow cytometry. Proteasome activity was determined using Proteasome-Glo™ Chymotrypsin-Like Cell-Based Assay (Promega). Luminescence was detected using a plate reader.

Results

LAMP2 isoform expression in human B lymphocytes

Lamp2c expression is regulated during morphogenesis in mice with some indication of tissue-specific distribution (20). Here, studies of the mRNA expression profile for the three isoforms in human tissues revealed that *LAMP2A* and *LAMP2B* were ubiquitously expressed while *LAMP2C* had a more restricted tissue distribution (Fig. 1B). Quantitative analysis of human spleen and muscle substantiated tissue-specific differences for *LAMP2C* compared with *LAMP2A* or *LAMP2B*, suggesting distinct regulation and possibly function for *LAMP2C* (Supplemental Fig 1B). Differences in isoform expression were also apparent in murine tissues (Supplemental Fig. 1C). LAMP-2A and LAMP-2B proteins are known to modulate autophagy pathways impacting B cell function and development (11, 19). To address whether differential regulation of *LAMP2* isoforms is observed in B cells, peripheral blood human B cells were stimulated with TLR7 or TLR9 agonists, R848 or CpG respectively. The mRNA encoding each *LAMP2* isoform was increased by TLR7 or TLR9 stimulation with expression strikingly higher in B cells stimulated with CpG (Fig. 1C). Of the three isoforms, the relative increase in *LAMP2C* expression was greatest with B cell TLR activation. Changes in *CD86* mRNA levels and IL-6 secretion confirmed B cell activation with R848 or CpG stimulation (Fig. 1C, 1D). Crosslinking of surface BCR also induces B cell activation and proliferation. *LAMP2* mRNA levels were evaluated in peripheral blood human B lymphocytes after BCR crosslinking, with the relative expression of each *LAMP2* isoform increasing upon B cell activation (Fig. 1E). Here, higher *CD86* mRNA levels corroborated B cell activation (Fig. 1E). Yet, in contrast to TLR activation,

with BCR crosslinking *LAMP2C* mRNA relative expression increased significantly less than *LAMP2A* or *LAMP2B* isoforms. These results suggest that expression of *LAMP2* isoforms may be distinctly regulated during B cell activation.

LAMP-2C expression impacts cytoplasmic Ag presentation via MHCII

The CMA translocator, LAMP-2A, regulates protein and peptide delivery from the cytoplasm into lysosomes. Overexpression of LAMP-2A but not LAMP-2B in human B cells increased cytoplasmic Ag presentation via MHCII (19). Given B cell activation by TLR ligands and BCR crosslinking can alter multiple steps in Ag processing and presentation including changes in co-stimulatory molecule expression and HLA-DM localization, ectopic expression of LAMP-2C was used to dissect the role of this lysosomal protein in Ag presentation. Human B lymphoblasts express low levels of *LAMP2C*, yet can be readily transfected with *LAMP2C* cDNA to promote higher ectopic expression (Supplemental Fig. 1D–F). *LAMP2A* and *LAMP2B* mRNA levels were unchanged with *LAMP2C* ectopic expression in B cells (Supplemental Fig. 1D, 1E). To assess the function of LAMP-2C in MHCII-restricted presentation, the B cell line PriessGAD expressing ectopic LAMP-2C (PG 2c) or transfected with a control vector (PG zeo) were analyzed for MHCII presentation of the cytoplasmic Ag glutamate decarboxylase (GAD), which relies upon CMA for epitope delivery to MHCII. Interestingly, MHCII presentation of cytoplasmic GAD Ag was decreased in cells overexpressing LAMP-2C (Fig. 2A). As a control, presentation of exogenous GAD_{273–285} peptide was tested, and no differences in T cell recognition were detected in LAMP-2C expressing cells (Fig. 2B). To further address the role of LAMP-2C, another human B- LCL, FrevSMA, was transfected with an empty vector (FS pCMV) or a plasmid encoding *LAMP2C* cDNA (FS 2c). FrevSMA cells express the cytoplasmic autoantigen SMA, a mutated form of Ig κ light chain trafficked to the cytosol for degradation. Translocation of SMA epitopes from the cytoplasm to MHCII also depends upon CMA (19). *LAMP2* isoforms mRNA and protein levels were analyzed to confirm ectopic expression of LAMP-2C in FS 2c cells (Supplemental Fig. 1E, 1F). Ectopic expression of LAMP-2C in these B cells diminished SMA autoantigen presentation by 10 fold compared with cells with wild-type levels of LAMP-2C (Fig. 2C). As a control, the presentation of exogenous κ _{188–203} peptide was evaluated, and no differences in peptide presentation were detected using B cells with ectopic LAMP-2C expression (Fig. 2D). Together, these data suggest that LAMP-2C inhibits the MHCII presentation of cytoplasmic Ags that rely upon the CMA pathway.

MHCII presentation of exogenous or membrane derived Ags was not altered by LAMP-2C expression

To gain a better understanding of the role of LAMP-2C in MHCII Ag presentation, B cells expressing LAMP-2C were incubated with exogenous protein Ags, human serum albumin (HSA) or tetanus toxoid (TT) which require endocytic uptake and processing to yield epitopes for MHCII. Presentation of HSA or TT epitopes derived from these exogenous Ags was unperturbed by changes in cellular LAMP-2C expression as assessed by T cell activation (Fig. 3A, 3B). Also, presentation of exogenously added HSA_{64–76} peptide or TT peptides was similar for control cells or cells with ectopic LAMP-2C expression (Fig. 3A, 3B). PriessGAD cells constitutively produce secretory Ig κ light chain, which serves as an

endogenous intravesicular source of Ag. For these Ig κ positive B cells, changes in LAMP-2C expression did not alter T cell recognition and responses to these membrane epitopes (Fig. 4A, 4B). The presentation of a viral membrane glycoprotein, hemagglutinin (HA), via MHCII was next examined. During influenza virus replication, newly synthesized HA molecules are trafficked through the Golgi compartments to reach the host cell membrane for virion formation and budding. Some HA molecules re-enter the endosomal network and are processed by acidic proteases, with the resulting epitopes binding MHCII molecules followed by the transit of these complexes to the surface for recognition by T cells. Ectopic expression of LAMP-2C in B cells did not diminish MHCII presentation of HA-derived epitopes as determined by T cell responses to virus-infected cells (Fig. 4C). To ensure optimal detection of changes in T cell responses, studies were carried out using a range of APC:T cell ratios (Fig. 4). These data suggest that MHCII presentation of Ags which enter cells via endocytosis or by the secretory pathway was not altered by changes in cellular LAMP-2C expression.

Alterations in cellular LAMP-2C levels failed to perturb MA in B cells

In B cells, MA sequesters cytoplasmic and nuclear Ags to deliver these molecules into autophagosomes for processing and subsequent MHCII presentation (23). Cellular stress including nutrient deprivation can up-regulate MA while blocking CMA (24). To examine if LAMP-2C expression modulates MA and Ag presentation via this pathway, B cells with and without ectopic LAMP-2C expression were transduced to express a fusion protein encoding the influenza matrix protein MP1 that is targeted to autophagosomes by the LC3 domain (22). These cells were co-culture with MP1 specific CD4⁺ T cells. Ectopic expression of LAMP-2C in PG 2c cells had no effect on MP1 Ag presentation (Fig. 5A). Autophagosome formation and turnover were monitored to further address whether cellular LAMP-2C levels impact MA. MA flux was evaluated by detecting changes in cellular LC3II levels +/- CQ. Similar LC3II protein levels detected in these cells indicate that MA was not altered by LAMP-2C expression (Fig. 5B). These results suggest that LAMP-2C affects the presentation of cytoplasmic Ags processed through CMA without altering MA.

Changes in LAMP-2C expression in B cells failed to alter the expression of key components of CMA and the MHCII pathway

LAMP-2 proteins reside primarily in lysosomes, with LAMP-2A regulating CMA and LAMP-2B playing a role in lysosome maturation (17, 25). Here we corroborated that cellular levels of molecules involved in CMA were not altered by the ectopic expression of LAMP-2C in B cells. Levels of the CMA substrates, GAD and SMA, were not disturbed in B cells ectopically expressing LAMP-2C (Fig. 6A, Supplemental Fig. 2A). Comparable levels of CMA chaperones HSP90 and HSC70 were observed in B cells expressing ectopic LAMP-2C or control cells (Fig. 6A). The expression of another lysosome-resident glycoprotein, LAMP-1, was unaltered with ectopic LAMP-2C in cells suggesting lysosome number and maturation were preserved (Fig. 6A). Immunoblotting analysis revealed that MHCII and Ii protein levels did not vary in B cells with ectopic LAMP-2C (Fig. 6B, Supplemental Fig. 2B). Furthermore, identical cell surface levels of the Ii peptide CLIP which associates with MHCII were detected by flow cytometry in these cells, suggesting that HLA-DM peptide editing function was not altered by LAMP-2C expression in B cells

(Fig. 6C). Another class II-like molecule, HLA-DO, functions as a modulator of HLA-DM (26). Consistent with the lack of change in CLIP expression, HLA-DO levels were unchanged with ectopic LAMP-2C expression in B cells (Fig. 6C, Supplemental Fig. 2C). Cell surface levels of MHCII molecules HLA-DR, -DQ, and -DP were also not altered in B cells overexpressing LAMP-2C (Fig. 6C, Supplemental Fig. 2C). Therefore, LAMP-2C expression does not affect cellular levels of well-established co-chaperones involved in CMA or key components of the MHCII pathway.

Studies have shown that lysosomal proteinases, such as cathepsin D (CatD), cathepsin B (CatB), and cathepsin L (CatL), play a role in MHCII-restricted presentation (27). These aspartyl and cysteinyl endoproteinases are required for Ag processing and Ii degradation (2, 3, 27). Differences in lysosomal proteinase levels can influence the peptide repertoire generated in lysosomal compartments, thus impacting the epitopes displayed for MHCII presentation. Ectopic expression of LAMP-2C in B cells did not alter cellular CatD subunit maturation or expression as detected in immunoblot assays (Fig. 7A). In addition, cells incubated with fluorogenic substrates specific for CatB or CatL revealed that the function of these cysteinyl endoproteinases was not altered by increased expression of LAMP-2C within lysosomal compartments (Fig. 7B). MHCII Ag presentation can also be facilitated by a lysosomal thiol reductase, gamma-interferon inducible lysosomal thiol reductase (GILT), which is abundantly expressed in professional APCs (8, 28). Immunoblotting analysis revealed that LAMP-2C overexpression in B cells did not interfere with GILT maturation or expression (Fig. 7A).

Some cytosolic Ags are dependent on the proteasome for MHCII-restricted presentation (29). Therefore, we investigated the ability of the proteasome to cleave a luminogenic chymotrypsin-like substrate as a means to quantitate the proteasome proteolytic activity in B cells expressing ectopic LAMP-2C. This functional assay indicated that the substrate was equally cleaved by the proteasome in B cell lines regardless of the level of cellular LAMP-2C (Fig. 7C). These results indicate that LAMP-2C, while negatively regulating Ag presentation via CMA, does not directly impair the function of Ag processing enzymes including cathepsins, GILT, and the proteasome.

Ectopic expression of LAMP-2C reduced the translocation of a cytoplasmic peptide to MHCII complexes

The delivery of peptides from the cytoplasm to vesicular MHCII molecules was enhanced in B cells with overexpression of the CMA receptor LAMP-2A (19). This led us to question if the transport of peptides from the cytoplasm to MHCII complexes was also influenced by cellular expression of LAMP-2C. A biotin-labeled GAD₂₇₃₋₂₈₅ peptide was introduced by electroporation into the cytoplasm of B cells expressing variable levels of LAMP-2C protein. Electroporation facilitates the delivery of antigenic peptides into the cytoplasm of B cells where these are selected by the CMA pathway for translocation into acidic endosomes or lysosomes (19). The translocated peptide can then be captured by MHCII molecules within these vesicular compartments. Strikingly, ectopic LAMP-2C expression in B cells reduced the abundance of newly formed MHCII-GAD peptide complexes, consistent with a role for LAMP-2C in blocking CMA (Fig. 8A). As a control, B cells were incubated with

peptides without electroporation which blocked cytoplasmic delivery and the formation of MHCII-peptide complexes (Fig. 8A). Consistent with this assay which detects epitope loading, MHCII presentation of the cytoplasmic GAD peptide was also reduced with ectopic LAMP-2C expression in B cells (Supplemental Fig.3A). To determine if the inhibitory effects of LAMP-2C on CMA could be overcome, a KFERQ motif which promotes HSC70 chaperone binding to CMA substrates was added to GAD peptide. Presentation of this modified peptide was still reduced in B cells ectopically expressing LAMP-2C (Supplemental Fig. 3A). Surprisingly, adding the KFERQ motif to GAD peptide also slightly diminished its presentation in B cells transfected with a control vector (Supplemental Fig. 3A). Similar results were seen with a control GAD peptide with an irrelevant AFERQ motif (Supplemental Fig. 3A).

Deficiencies in CMA can frequently manifest as the intracellular accumulation of specific proteins which typically transit via this pathway into lysosomes for degradation. Immunoblotting analysis demonstrated increased cellular levels of two additional well-established CMA substrates, phosphorylated I κ B α (p-I κ B α) and RNase A (30, 31), in B cells with ectopic LAMP-2C expression pointing again to disruptions in CMA (Fig. 8B). Complexes of the chaperone HSC70 and CMA substrates can be detected in cells consistent with the formation of distinct transient complexes which promote substrate membrane translocation into lysosomes (12). The association of HSC70 with one of its substrates, cytoplasmic GAD, decreased suggesting that LAMP-2C likely perturbs the stability of these chaperone-substrate protein complexes required for CMA (Supplemental Fig. 3B). These findings suggest that increased expression of LAMP-2C in B cells obstructs chaperone association and the translocation of cytoplasmic peptides and proteins to selectively disrupt MHCII Ag presentation via CMA. Thus, LAMP-2C acts as a negative regulator of CMA in B lymphocytes.

Discussion

The current study examines the expression and a novel function of the conserved lysosomal membrane glycoprotein LAMP-2C in B lymphocytes MHCII Ag presentation. Like its homologous counterparts *LAMP2A* and *LAMP2B*, *LAMP2C* mRNA expression increased with B cell activation in response to TLR ligands or BCR crosslinking. Multiple gene products critical to Ag presentation are upregulated with B cell activation, and thus ectopic expression of LAMP-2C in B lymphoblasts was used to specifically explore the functions of this LAMP-2 isoform. Presentation of exogenous peptides and Ags was unaltered in B cells with increased LAMP-2C expression. Consistent with this finding, there was no change in MHCII surface expression or lysosomal protease activity in B lymphoblasts with increased LAMP-2C levels. T cell recognition of epitopes derived from membrane Ags or from an Ag that relies on MA was also unperturbed in B cells with enhanced LAMP-2C expression. By contrast, MHCII presentation of select cytoplasmic Ags via the CMA pathway was significantly diminished following LAMP-2C ectopic expression in B lymphoblasts. These results suggest LAMP-2C functions as a highly specific negative regulator of CMA in B lymphocytes.

LAMP-2A and -2B are constitutively expressed in most tissues and cells, but the mechanisms regulating each LAMP-2 isoform expression are not well understood. The global detection of these lysosomal proteins may be linked to the critical requirement for these isoforms in CMA and MA, processes important in tissue and cellular development, stress responses, and host immunity (9–11). LAMP-2A expression is essential for CMA and diminishes with aging and oxidative stress (14, 15, 25). LAMP-2B modulates lysosome maturation, with the absence of this protein slowing phagosome fusion and driving autophagosome accumulation (17). By contrast, LAMP-2C displays a unique profile of tissue-specific expression as shown here for humans and mice, as well as in published studies with rodent cells and organs (20). Quantitative analysis revealed human *LAMP2A* and *LAMP2B* mRNA levels were comparable in spleen and muscle, while *LAMP2C* message was significantly lower in spleen. By contrast, the expression of each isoform in murine tissues was reduced in the spleen compared with muscle or heart. Of note, cardiac and muscle defects are clinical hallmarks of Danon's disease, a disorder linked to mutations in one or more of the human LAMP-2 isoforms (17). Low levels of *LAMP2C* mRNA were detected here in human B lymphocytes and by others in murine macrophage lines, suggesting a possible role in antigen recognition or clearance (20). The induction of *LAMP2C* with B cell activation in the current study also points to a potential role in immunity. B cell treatment with the TLR7 ligand R848 or TLR9 ligand CpG increased the mRNA expression of each *LAMP2* isoform, although the fold increase was greatest for *LAMP2C*. BCR ligation also enhanced the expression of each *LAMP2* isoform, with the greatest increase in *LAMP2A* and *LAMP2B* messages compared with *LAMP2C*. These results suggest specificity in the pathways leading to *LAMP2C* induction during B cell activation and potential roles for LAMP-2 isoforms in B cell engagement with Ags or pathogens.

B cell activation exerts multiple effects on Ag presentation pathways including increasing the expression of co-stimulatory and MHCII molecules, promoting the transit of HLA-DM to endosomal compartments, transporting the BCR to endosomes and autophagosomes, and promoting cytokine production which can impact T cell responses (26, 32, 33). Functional analyses of T cell recognition in the current study revealed LAMP-2C selectively impacts MHCII Ag presentation by blocking CMA and the acquisition of antigenic epitopes via this pathway. LAMP-2A functions as part of a translocator complex in CMA, moving proteins and peptides from the cytoplasm into lysosomes for processing. Increasing LAMP-2A expression in B cells enhanced MHCII presentation of several cytoplasmic autoantigens (19). This pathway for Ag presentation was also dependent on two important cytoplasmic chaperones, HSC70 and HSP90, which are required for CMA (19, 34). While LAMP-2A ectopic expression enhanced the translocation of cytoplasmic peptides into lysosomes for MHCII display (19), here LAMP-2C expression blocked cytoplasmic peptide translocation and MHCII presentation. LAMP-2C expression did not perturb MHCII presentation of exogenous and membrane Ags or Ag delivered by MA. Direct analysis of cellular levels of MA flux also indicated this process was not impaired with increased LAMP-2C expression. These results indicate LAMP-2C is not an inhibitor of LAMP-2B functions, as this latter isoform regulates lysosome fusion and maturation as well as the formation of autophagosomes (17). Thus, the induction of *LAMP2C* during B cell activation may alter the

profile of cytoplasmic but not extracellular antigenic epitopes displayed by MHCII through effects on CMA.

LAMP-2 isoforms differ predominantly in their C-terminal cytoplasmic tail domains with more conserved substitutions in their transmembrane and membrane proximal domains. The highly conserved glycosylated domain of each isoform extends into the lysosome lumen of most cells, and LAMP-2 is frequently used as a marker for lysosomes. However, upon T cell activation and in some tumors, LAMP-2 isoforms are delivered to the cell surface as secretory lysosomes fuse with the plasma membrane. By contrast, we have been unable to detect significant cell surface LAMP-2 expression in B cells (19). Each of the LAMP-2 isoforms contains two cytoplasmic tail tyrosine motifs (YXXG and GYXX) important for membrane residence in lysosomes. LAMP-2 isoforms also contain two conserved glycine residues in their transmembrane domain, with studies of LAMP-2A indicating these are important in oligomerization required for CMA (35, 36). Whether mixed oligomers of LAMP-2 isoforms exist within lysosomes has not been determined, but could explain the ability of LAMP-2C to disrupt CMA and peptide translocation. In contrast with LAMP-2A, a peptide from the tail of LAMP-2C does not bind the CMA chaperone HSC70 (20). However, studies here revealed impaired association of HSC70 with the CMA substrate GAD in cells with increased LAMP-2C expression. These results suggest mechanistically LAMP-2C may alter chaperone capture of substrates for CMA translocation. Repeated attempts to detect the association of LAMP-2C with other LAMP-2 isoforms have not been successful in human B cells, although transient complex formation cannot be ruled out. Recently, LAMP-2C has been implicated in transporting DNA and RNA from the cytoplasm into lysosomes of cells (20, 21). This finding was based upon the ability of the cytoplasmic tail of LAMP-2C to bind nucleic acids and analysis of HeLa cells expressing LAMP-2C (20, 21). Positively charged residues in the tail of LAMP-2C were implicated in nucleic acid binding, but a very similar motif of four positively charged residues is found in LAMP-2B which does not promote lysosomal nucleic acid transport. Additional studies are needed to define at a molecular level precisely how LAMP-2C disrupts CMA and whether nucleic acids play a role in this process.

Disruption of LAMP-2A and CMA in T cells revealed that this pathway regulates cellular levels of Itch, an ubiquitin ligase, and the calcineurin inhibitor RCAN1 likely by blocking their degradation (18). In B cells with increased LAMP-2C expression, the levels of two proteins which are targeted for degradation by CMA, RNase A and p-I κ B α were significantly increased, again consistent with a block in this autophagy pathway. No difference in the proliferation or survival of B cells was detected with LAMP-2C expression. These latter results suggest a novel role for LAMP-2C in regulating CMA, and together with the work in T cells, the importance of this selective autophagy pathway in lymphocyte functions.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations used in this article

LAMP-2	lysosome-associated membrane protein-2
CMA	chaperone-mediated autophagy
MA	macroautophagy
MHCII	MHC class II

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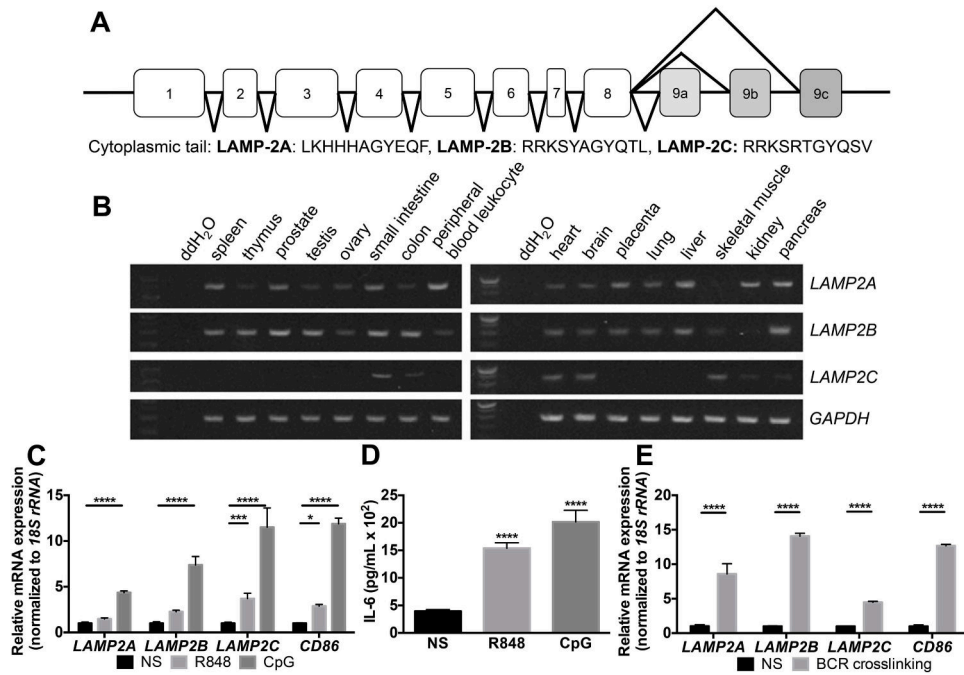


Figure 1. *LAMP2* isoform expression and regulation during B cell activation

(A) Exon structure and alternative splicing of human *LAMP2*. The three isoforms have an identical luminal domain but distinct transmembrane and cytoplasmic domains. (B) RT-PCR analysis for *LAMP2* isoforms in human tissues. *LAMP2A* and *LAMP2B* are ubiquitously expressed while *LAMP2C* is tissue specific. (C) Peripheral blood human B cells were treated for 24 h with R848, CpG, or left untreated (NS). Gene expression of *LAMP2* isoforms and *CD86* were analyzed by qPCR. (D) B cell TLR7 or TLR9 stimulation was detected via IL-6 release. (E) Peripheral blood B cells were stimulated for 24 h to crosslink BCR or left untreated (NS). *LAMP2* isoforms and *CD86* mRNA were detected as in (C). Data were analyzed by two-way ANOVA (C and E) or by one-way ANOVA (D). * $p < 0.05$, *** $p < 0.001$, and **** $p < 0.0001$ (mean \pm SD, $n = 3$).

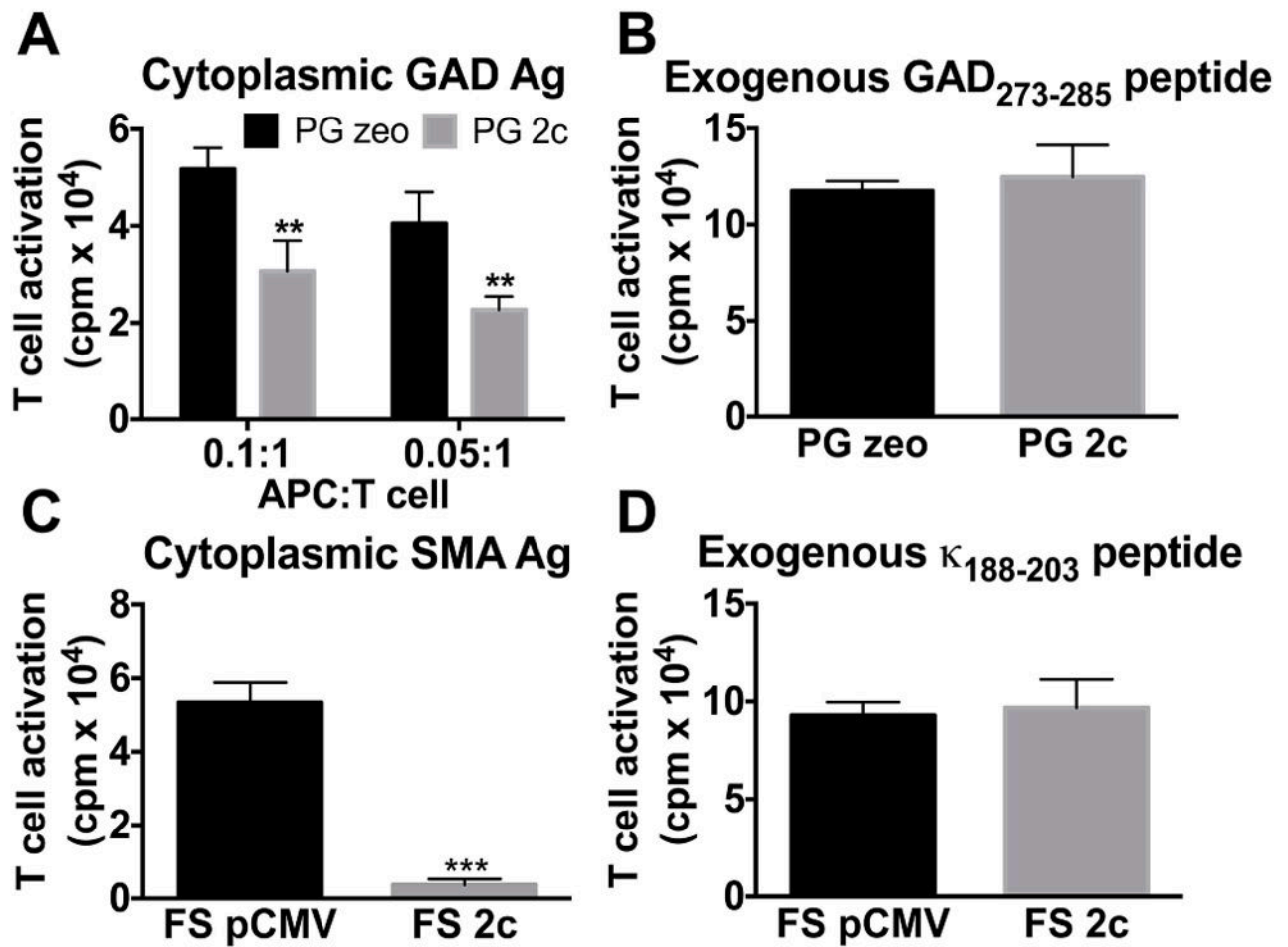


Figure 2. Inhibition of MHCII cytoplasmic Ag presentation in B cells ectopically expressing LAMP-2C

(A) PG zeo and PG 2c B cells with ectopic LAMP-2C were incubated with GAD-specific T cells to analyze T cell activation. (B) As a control, PG zeo and PG 2c B cells were incubated with 10 μ M of GAD₂₇₃₋₂₈₅ peptide for 4 h and cultured with GAD-specific T cells at APC:T cell ratio of 0.05:1 to monitor MHCII presentation. (C) FS pCMV and FS 2c B cells were incubated with κ -specific T cells at APC:T cell ratio of 1:1 to detect T cell activation. (D) FS pCMV and FS 2c B cells were incubated overnight with 10 μ M of $\kappa_{188-203}$ peptide and cultured with κ -specific T cells at APC:T cell ratio of 1:1 to measure T cell activation. Data were analyzed by two-way ANOVA (A) or by two-tailed, unpaired Student *t* test (C). ***p* < 0.01 and ****p* < 0.001 (mean \pm SD, representative of *n* 3).

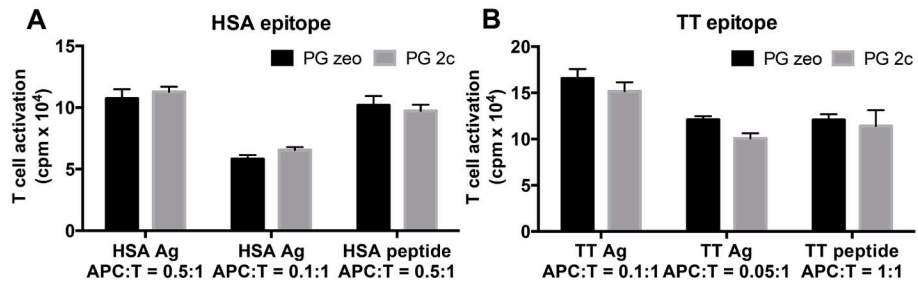


Figure 3. LAMP-2C expression did not alter the MHCII presentation of exogenous Ags
(A) B cells +/- ectopic LAMP-2C expression were incubated overnight with 20 μ M of HSA Ag or 4 h with 10 μ M HSA₆₄₋₇₆ peptide and then cultured with HSA-specific T cells to measure T cell activation. **(B)** PG zeo and PG 2c B cells were incubated overnight with 0.1 μ M of TT Ag or for 4 h with 0.2 μ M of TT peptides. APCs were cultured with TT-specific T cells to analyze T cell activation. (mean \pm SD, representative of n 3).

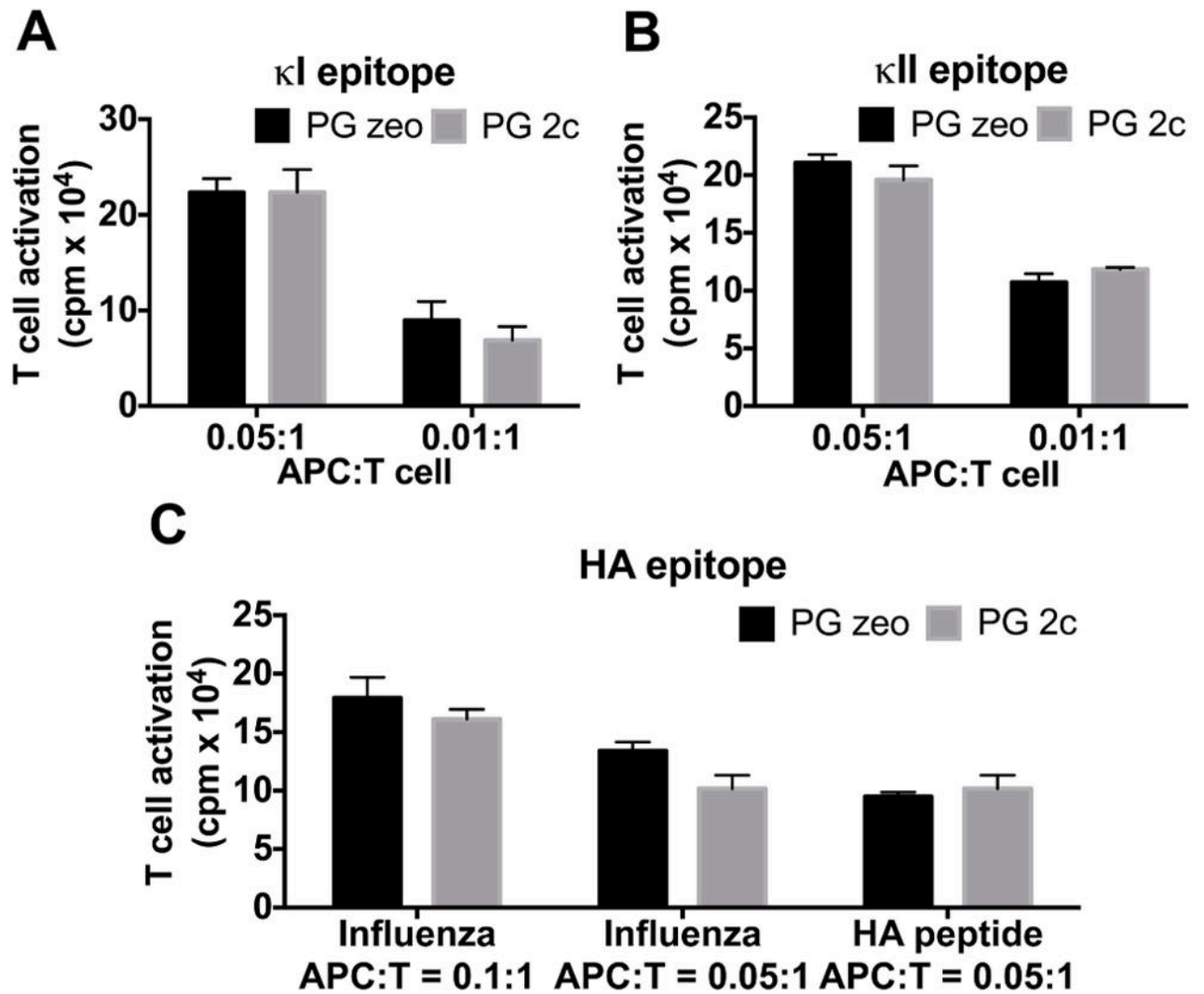


Figure 4. MHCII presentation of self or viral membrane proteins was not affected by LAMP-2C overexpression

(A and B) PG zeo and PG 2c B cells were cultured with κ I- or κ II-specific T cells. T cell activation was monitored to analyze MHCII presentation of membrane Ag, Ig κ . (C) PG zeo and PG 2c B cells were infected overnight with influenza A X-31, A/Aichi/68 (H3N2) or incubated with HA₃₀₇₋₃₁₉ peptide for 4 h and then cultured with HA-specific T cells to measure T cell activation. (mean \pm SD, representative of n 3).

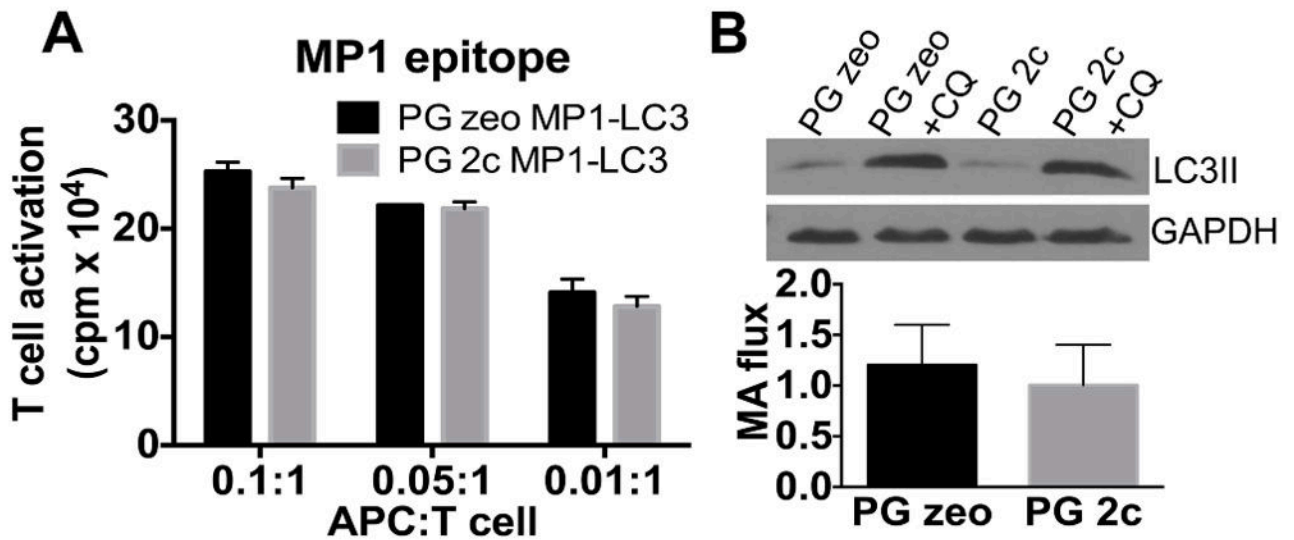


Figure 5. Macroautophagy was not altered with B cell ectopic LAMP-2C expression

(A) PG zeo and PG 2c B cells were transduced to express the chimeric Ag MP1-LC3. MP1-LC3 is targeted to autophagosomes by the LC3 domain. APCs were cultured with MP1-specific T cells to monitor T cell activation. (B) PG zeo and PG 2c B cells were incubated overnight +/- 20 μ M CQ, an inhibitor of lysosome acidification, to monitor autophagosome formation and turnover. MA flux was evaluated by immunoblotting to detect changes in cellular LC3II levels +/- CQ. (mean \pm SD, representative of n = 2–3).

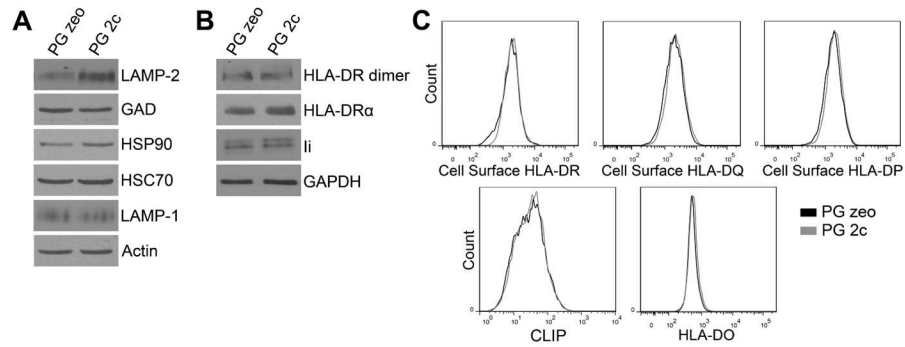


Figure 6. Changes in cytoplasmic MHCII-restricted presentation were not due to differential expression of proteins required for Ag presentation or CMA

(A and B) Lysates from PG z80 and PG 2c B cells were resolved by SDS-PAGE and immunoblotted to detect LAMP-2, GAD, HSC70, HSP90, LAMP-1, HLA-DR dimer, HLA-DR α chain, Ii, actin, and GAPDH. (C) PG z80 and PG 2c B cells were incubated with Abs to detect cell surface expression of HLA-DR, HLA-DQ, HLA-DP, or CLIP and total cellular levels of HLA-DO. (mean \pm SD, representative of n 4).

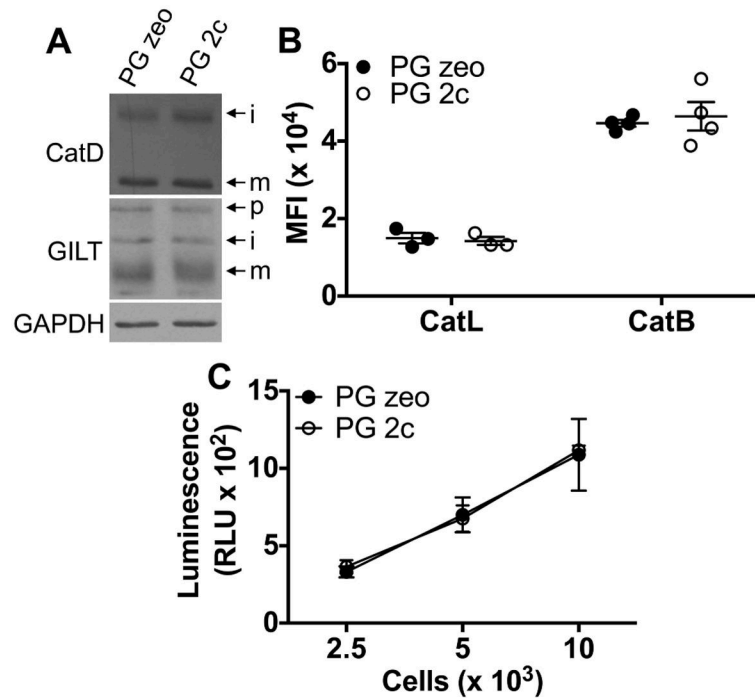


Figure 7. Cellular lysosomal enzyme levels and proteolytic processing by cathepsins and the proteasome were unaffected by ectopic expression of LAMP-2C in B cells

(A) Comparable maturation of CatD and GILT in B cells expressing LAMP-2C. Lysates from PG zeo and PG 2c B cells were resolved by SDS-PAGE and immunoblotted to detect the precursor (p), intermediate (i) or mature (m) form of CatD or GILT. (B) To analyze CatL or CatB activities, PG zeo and PG 2c B cells were incubated for 30 min at 37°C with membrane permeable fluorogenic substrates specific for CatL or CatB. Enzyme activity was detected by flow cytometry. (C) Proteasome activity was determined by incubating PG zeo and PG 2c B cells with a luminogenic chymotrypsin-like substrate. (mean ± SD, representative of n = 2–4).

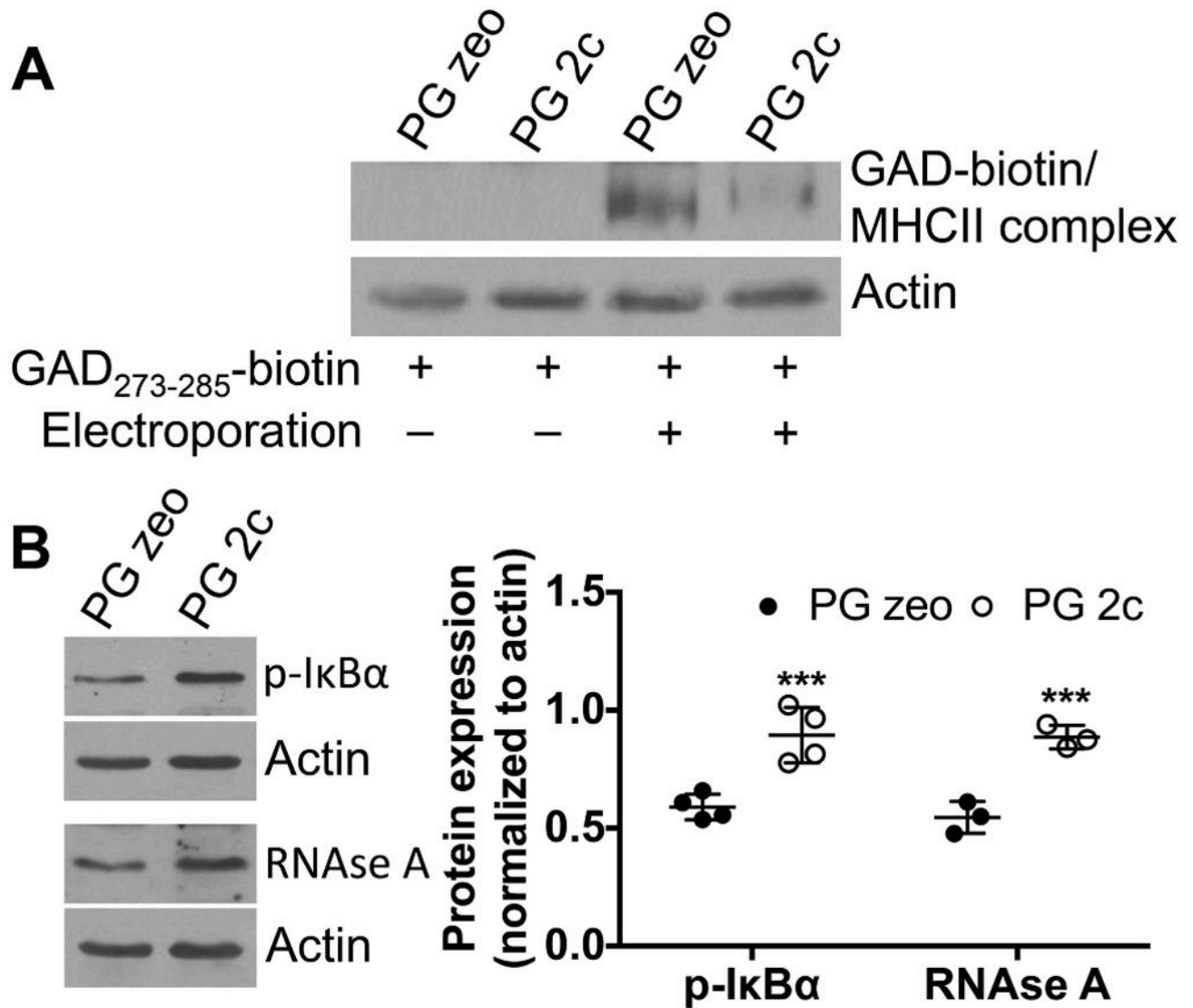


Figure 8. Decreased peptide translocation from the cytoplasm to endosomal MHCII molecules and reduced processing of CMA substrates

(A) PG zeo and PG 2c B cells were incubated with 2 mM GAD₂₇₃₋₂₈₅-biotin and electroporated to deliver this peptide to the cytoplasm. Control cells were not subjected to electroporation. Cells were acid-stripped, cultured 16 h, and lysates resolved by SDS-PAGE. Streptavidin-HRP was used to detect biotin-peptide complexed with MHCII molecules. Immunoblotting of actin was used as a loading control. (B) Basal levels of CMA substrates p-IkBa and RNase A were evaluated by immunoblotting. Data were analyzed by two-way ANOVA. *** $p < 0.001$ (mean \pm SD, $n = 3-4$).